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REMARKS

Applicants have carefully studied the Office Action mailed on April 6, 2004, which issued in connection with the above-identified application. The present response is intended to be fully responsive to all points of rejection raised by the Examiner and is believed to place the claims in condition for allowance. Favorable reconsideration and allowance of the present claims are respectfully requested.

Pending Claims

Claims 2-3, 11-16, 29, 48-51, 54, and 56-65 were pending and at issue in the application. Claims 2, 3, 11-16, 48-51, 54, 56, 60, 61, and 63-65 have been rejected under 35 U.S.C. §112, first paragraph, for lack of enablement and/or written description. Claims 2, 3, 11-14, 48-51, 54, and 63-65 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite. Claims 2, 3, 29, 48-50, 54, and 58 have been rejected under 35 U.S.C. §102(b) as being anticipated by prior art. Claims 2, 3, 48, and 54 have been rejected under 35 U.S.C. §103(a) as being obvious over prior art.

Claims 2, 3, 13-15, 48-50, 54, 64, and 65 have been canceled without prejudice or disclaimer. Claim 16 has been amended to correct dependency. Claims 51 and 63 have been amended to delete the recitation "detecting the absence of expression of a CASP8 protein, and detecting the absence of a *CASP8* mRNA". Claims 29 and 58 have been amended to by adding the recitation "*CASP8* gene-specific" to refer to oligonucleotide PCR primers for amplification of at least a part of the 5' untranslated region of *CASP8* genomic DNA. Support for this recitation can be found, for example, at page 3, lines 10-23; page 7, line 29 - page 8, line 10; page 12, lines 16-19; page 29, line 26 - page 30, line 3; page 30, line 25 - page 31, line 4, and Example 3 (especially page 52, lines 12-26) of the specification. No new subject matter has been added as a result of these amendments, no new search is required, and no new issues are raised.

Upon entry of the above-identified amendments, claims 11, 12, 16, 29, 51, and 56-63 will be pending.

35 U.S.C. § 112, Second Paragraph Rejections

In the Action (Section 10), claims 2, 3, 11-14, 48-51, 54, and 63-65 stand rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner contends that the phrases "detecting the absence of expression of a CASP8 protein" and "detecting the absence of a *CASP8* mRNA" are indefinite, because the specification provides no structural definition of CASP8 protein or *CASP8* mRNA.

As claims 2, 3, 13-14, 48-50, 54, and 64-65 have been canceled, the rejection of these claims is rendered moot. Independent Claims 51 and 63 have been amended to delete the recitation "detecting the absence of expression of a CASP8 protein, and detecting the absence of a *CASP8* mRNA". Claims 11 and 12 depend from claims 51 and 63. Accordingly, with respect to claims 11, 12, 51, and 63, the rejection is also rendered moot.

However, applicants note for the record that the amendment of claims 51 and 63 is made solely to expedite the prosecution and not as an admission of indefiniteness of the phrases "detecting the absence of expression of a CASP8 protein" and "detecting the absence of a *CASP8* mRNA". Applicants reserve the right to pursue this subject matter in a continuing application.

35 U.S.C. § 112, First Paragraph Rejections

In the Action (Section 11), claims 11-16, 51 and 63-65 stand rejected under 35 U.S.C. §112, first paragraph, for lack of enablement and written description. Specifically, the Examiner contends that the specification, while being enabling for methods of prognosis of neuroblastoma

comprising the detection of methylation of *CASP8* gene, does not reasonably provide enablement for methods of diagnosis or prognosis comprising the detection of the absence of CASP8 protein or *CASP8* mRNA, because it fails to define the scope of the terms "CASP8 protein" and "*CASP8* mRNA". The Examiner further contends that the disclosure does not contain an adequate written description, examples or guidance by which methods comprising the detection of CASP8 protein or *CASP8* mRNA could be placed in the hands of the skilled artisan.

Claims 2, 3, 11-16, 48-51, 54, and 63-65 stand further rejected (Section 12 of the Office Action) under 35 U.S.C. §112, first paragraph, for lack of adequate written description of the genus "*CASP8* mRNA" and the genus "CASP8 protein". The Examiner contends that the specification fails to contain a written description of a sufficient number of the members of either genus.

As claims 2, 3, 13-15, 48-50, 54, and 64-65 have been canceled, the rejection of these claims is rendered moot. Independent claims 51 and 63 have been amended to delete the recitation "detecting the absence of expression of a CASP8 protein, and detecting the absence of a *CASP8* mRNA". Claims 11, 12 and 16 depend from claims 51 and 63. Accordingly, with respect to claims 11, 12, 16, 51, and 63, the rejections based on the recitation "detecting the absence of expression of a CASP8 protein, and detecting the absence of a *CASP8* mRNA" are also rendered moot.

However, as stated above, the amendment of claims 51 and 63 is made solely to expedite the prosecution and not as an admission of lack of enablement or adequate written description of the phrases "detecting the absence of expression of a CASP8 protein" and "detecting the absence of a *CASP8* mRNA". Applicants reserve the right to pursue this subject matter in a continuing application.

In Section 11 of the Office Action, the Examiner also contends that, while the data provided in the present application appear to demonstrate that detection of *CASP8* gene

methylation alone or in combination with *CASP8* gene deletion may be a useful indicator of poor prognosis for neuroblastoma, the data do not extrapolate to all other cancers. Applicants respectfully disagree and note that the present specification discloses that any form of cancer can be evaluated using the diagnostic methods of the invention (see, *e.g.*, page 28, lines 28-29). At page 9, lines 2-22, the present specification provides multiple examples of cancers that, in addition to neuroblastoma, can be diagnosed (or their prognosis determined) by detecting inactivation of *CASP8* gene using the methods of the present invention. As further specified at page 9, lines 18-22 and page 28, line 29 - page 29, line 2, preferred cancers which can be evaluated using the diagnosis or prognosis methods of the present invention include, in addition to neuroblastoma, small cell lung carcinoma, non-small cell lung carcinoma, colorectal carcinoma, and uterine cervical carcinoma. Indeed, as disclosed in Shivapurkar *et al.* article published after the filing date of the present application (Cancer Biology & Therapy, 1: 65-69, 2002; attached as Exhibit A), *CASP8* gene expression was lost in 79% of small cell lung carcinoma cell lines with 59% of expression loss associated with *CASP8* promoter methylation. This article also discloses that *CASP8* promoter methylation was observed in 35% of small cell lung carcinoma tumors and 18% of bronchial carcinoids (see Abstract). As reported in Harada *et al.* article published after the filing date of the present application (Cancer Research, 62: 5897-5901, 2002; attached as Exhibit B), frequent *CASP8* gene methylation was detected, in addition to neuroblastomas, in rhabdomyosarcomas (83%), medulloblastomas (81%), and retinoblastomas (59%) (see Abstract). In the last paragraph of this article, the authors conclude that "the loss of protein expression and enzymatic activity of the *CASP8* gene are common in pediatric tumor cell lines".

In the Office Action (Section 13), claims 11, 12, 15, 48, 51, 54, 56, 60, 61, and 63 have been also rejected under 35 U.S.C. §112, first paragraph, for lack of enablement of any methods of detection of DNA methylation other than methylation PCR.

As claims 15, 48 and 54 have been canceled, the rejection of these claims is rendered moot. With respect to the remaining claims, applicants respectfully traverse the rejection and note that the present specification provides a detailed disclosure of methylation PCR simply to identify it as a preferred method but by no means as the only available method for determining methylation of *CASP8* genomic DNA. Various methods for detecting DNA methylation other than methylation PCR were well known in the art at the time the present application was filed and therefore the disclosure of such methods was not required. For example, Herman *et al.* reference (Proc. Natl. Acad. Sci. USA, 93: 9821-9826, 1996) cited in the present specification (see, *e.g.*, page 54, lines 4-5) and incorporated by reference in its entirety discloses several well-established methods for determination of DNA methylation such as (i) Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences that contain one or more methylated CpG sites; (ii) combined use of methylation-sensitive restriction enzymes and PCR, when, after digestion of DNA with a restriction enzyme, PCR amplifies the fragment flanking the restriction site only if DNA cleavage has been prevented by methylation, and (iii) the chemical modification of cytosine to uracil by bisulfite treatment followed by amplification and sequencing (see page 9821 of Herman *et al.*).

Applicants further respectfully submit that, by requiring detailed experimental data for cancers other than neuroblastoma and by requiring disclosure for various methods for determining methylation of *CASP8* genomic DNA, in addition to methylation PCR, the Examiner imposes an overly high and burdensome duty on applicants, one not required by Section 112 or by the case law[1]. Thus, according to the current law and patent practice, the specification can permit some inferences to be drawn by those skilled in the art, and still comply

[1] *See*, in particular, *In re Wands*, 858 F.2d 731-40, 8 USPQ2d at 1400-07 (Fed. Cir. 1988)

with the enablement and written description requirement. In other words, there is no requirement that the claims be restricted to the working examples. Section 2164.03 of MPEP recites:

> the scope of the required enablement varies inversely with the degree of predictability involved, but even in unpredictable arts, a disclosure of every operable species is not required (*In re Vaeck*, 947 F.2d 488, 496 & n.23, 20 USPQ2d 1438, 1445 & n.23 (Fed. Cir., 1991); *In re Vickers*, 141 F.2d 522, 526-27, 61 USPQ 122, 127 (CCPA 1944); *In re Cook*, 439 F.2d 730, 734, 169 USPQ 298, 301 (CCPA 1971))

As further stated in section 2164.08 of MPEP:

> claims are not rejected as broader than the enabling disclosure under 35 U.S.C. 112 for non-inclusion of limitations dealing with factors which must be presumed to be within the level of ordinary skill in the art; the claims need not recite such factors where one of ordinary skill in the art to whom the specification and claims are directed would consider them obvious (*In re Skrivan*, 427 F.2d 801, 806, 166 USPQ 85, 88 (CCPA 1970))... When analyzing the enabled scope of a claim, the teachings of the specification must not be ignored because claims are to be given their broadest reasonable interpretation that is consistent with the specification.

See also *Application of Angstadt*, 537 F.2d 498, 502-503, 190 USPQ 214, 218 (CCPA 1976) (applicants "are not required to disclose every species encompassed by their claims even in an unpredictable art"). Similarly, in *In re Rasmussen*, court stated that "a claim may be broader than the specific embodiment disclosed in a specification." 650 F.2d 1212, 1215, 211 USPQ 323, 326 (CCPA 1981). Finally, in *In re Goffe*, the court stated:

> To provide effective incentives, claims must adequately protect inventors. To demand that the first to disclose shall limit his claims to what he has found will work or to materials which meet the guidelines specified for "preferred" materials in a process such as the one herein involved would not serve the constitutional purpose of promoting progress in the useful arts.

542 F.2d 564, 567, 191 USPQ 429, 431 (CCPA 1976).

In light of these standards and the above-presented arguments, it is believed that the present application provides an adequate enablement and written description for methods of prognosis and diagnosis for cancers other than neuroblastoma and for methods of detecting *CASP8* genomic DNA methylation other than methylation PCR.

In the Action (Section 11), the Examiner further contends that the specification, while being enabling for methods of prognosis comprising the detection of methylation of *CASP8* gene, does not reasonably provide enablement for methods of diagnosis comprising the detection of methylation of *CASP8* gene. Specifically, the Examiner contends that it is not clear how measurement of *CASP8* gene methylation alone or in combination with *CASP8* gene deletion would serve as a diagnostic method, because the specification fails to teach what other possible diagnosis one of skill in the art would be differentiating from. In response, applicants respectfully submit that the term "diagnosis" as commonly used in the art and defined in the present specification (see page 28, lines 21-22) does not assume that one would be distinguishing between more than one possible condition. To diagnose a disease, one simply needs to distinguish between a healthy ("normal") state and a disease state (see an example of a standard dictionary definition of the term "diagnosis" attached as Exhibit C). The present specification satisfies this definition, *e.g.*, by providing that *CASP8* gene methylation serves as a sign of the loss of *CASP8* gene function (*i.e.*, inactivation of the *CASP8* gene) which, in turn, may lead to deregulation of cellular apoptosis and development of a cancer (see, *e.g.*, the disclosure provided at page 3, lines 6-8; page 28, line 15 - page 29, line 2, and page 61, lines 1-7).

In light of the above-presented amendments and arguments, the rejections under 35 U.S.C. §112, first paragraph, are believed to be overcome and withdrawal of such is kindly requested.

35 U.S.C. § 102 and 103 Rejections

In the Office Action (Section 9), the Examiner has maintained the rejection of claims 2, 3, 48-50, and 54 under 35 U.S.C. §102(b) as being anticipated by PCT Application No. WO 97/46662 by Dixit *et al.* The Examiner contends that, Dixit *et al.* application teaches methods for detecting the absence of CASP8 (ICE LAP-7/FLICE) protein and detecting the absence of CASP8 mRNA.

As claims 2, 3, 48-50, and 54 have been canceled, the rejections of these claims is rendered moot.

Claims 2, 3 and 48 have been also rejected (Section 14 of the Office Action) under 35 U.S.C. §103(a) as being obvious over Mandruzatto *et al.* (J. Exp. Med., 186: 785-793, 1997). The Examiner contends that it would been *prima facie* obvious to use the teachings of Mandruzatto *et al.* (that a *CASP8* gene mutation produces a protein that impairs apoptosis) to make a biochemical or immunological method for the detection of the absence of a functional CASP8 tetramer in a primary cancer cell as a measure of inactivation of the *CASP8* gene.

As claims 2, 3 and 48 have been canceled, the rejections of these claims is rendered moot.

Claims 2, 3, 48, and 54 have been also rejected (Section 15 of the Office Action) under 35 U.S.C. §103(a) as being obvious over Scaffidi *et al.* (J. Biol. Chem., 272: 26953-8, 1997) in view of Mandruzatto *et al.* The Examiner contends that Scaffidi *et al.* reference teaches that a small cell lung carcinoma cell line is negative for FLICE (CASP8) expression and concludes that one would have had a reasonable expectation of success in using the method of Scaffidi *et al.* in a primary cancer cell, because Mandruzatto *et al.* reference teaches that the lack of CASP8 expression likely confers a selective advantage on tumor cells.

As claims 2, 3, 48, and 54 have been canceled, the rejection of these claims is rendered moot.

In the Office Action (Section 16), claims 29 and 58 have been rejected under 35 U.S.C. §102(b) as being anticipated by Boehringer Mannheim random hexamer mixtures (1997 Catalog No. 1 277 081). The Examiner contends that claims 29 and 58 read on random hexamer mixtures of Boehringer Mannheim, because the primers are to be used for the amplification of at least a part of the 5' untranslated region or for the amplification of sequences that comprise either SEQ ID NO: 1 or SEQ ID NO: 2.

In response, applicants respectfully note that, as specified at page 3, lines 10-23; page 7, line 29 - page 8, line 10; page 12, lines 16-19; page 29, line 26 - page 30, line 3; page 30, line 25 - page 31, line 4, and Example 3 (especially page 52, lines 12-26) of the present application, the oligonucleotide primers used for the amplification of at least a part of the 5' untranslated region or for the amplification of sequences that comprise either SEQ ID NO: 1 or SEQ ID NO: 2 are *CASP8* gene-specific oligonucleotides and not random hexamers as present in the mixture sold by Boehringer Mannheim. As recited in claim 29, the oligonucleotide primers are used in a methylation polymerase chain reaction (PCR) assay. The methylation PCR relies on the use of sequence-specific PCR primers to amplify a DNA region suspected of being methylated. Indeed, Herman *et al.* reference (Proc. Natl. Acad. Sci. USA, 93: 9821-9826, 1996) cited in the present application (see, *e.g.*, page 54, lines 4-5) and incorporated by reference in its entirety specifies at page 9823 (section entitled "Primers Design for MSP") that:

> Primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified... The multiple mismatches in these primers, which are specific for these different types of DNA, suggest that each primer set should provide amplification only from the intended template.

In contrast to the sequence-specific oligonucleotide primers disclosed in the present application and recited in the present claims, the random hexanucleotide primers sold by

Boehringer Mannheim do not allow the sequence-specific amplification of at least a part of the 5' untranslated region or for the amplification of sequences that comprise either SEQ ID NO: 1 or SEQ ID NO: 2. If used in a PCR reaction, these random primers will hybridize to all possible DNA sequences present in a sample and therefore may only allow non-specific amplification of all of these sequences. In fact, as provided in a product information sheet for the Boehringer Mannheim mix (presently Roche Catalog No. 1 277 081; attached as Exhibit D), these random hexanucleotide primers are not even recommended for the use in PCR reactions, but are recommended for use as labeled probes for screening gene libraries, Southern and Northern blots, and *in situ* hybridization.

It follows, that, in contrast to the Examiner's assertion, claims 29 and 58 do not read on random hexamer mixtures of Boehringer Mannheim. To further clarify the differences between the oligonucleotide primers used in the kits of the present invention and random hexanucleotides, claims 29 and 58 have been amended to recite "*CASP8* gene-specific oligonucleotides".

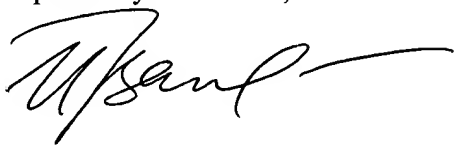
In light of the above-presented amendments and arguments, the anticipation and obviousness rejections are believed to be overcome and withdrawal of such is kindly requested.

CONCLUSION

Applicants request entry of the foregoing amendments and remarks in the file history of this application. In view of the above amendments and remarks, it is respectfully submitted that claims 11, 12, 16, 29, 51, and 56-63 are now in condition for allowance and such action is earnestly solicited. If the Examiner believes that a telephone conversation would help advance the prosecution in this case, the Examiner is respectfully requested to call the undersigned agent

at (212) 527-7634. The Examiner is hereby authorized to charge any additional fees associated with this response to our Deposit Account No. 04-0100.

Respectfully submitted,



Dated: July 6, 2004

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Research Article

Differential Inactivation of Caspase-8 in Lung Cancers

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ABSTRACT

Caspase-8 (CASP8) is an apoptosis inducing cysteine protease which is activated through the formation of a death-inducing signaling complex when death receptors are complexed to their specific ligands. Recent reports indicate that CASP8 expression is lost via a combination of promoter methylation and allelic loss in a subset of neuroblastomas. We investigated the state of the gene in lung tumors and cell lines. RT-PCR studies indicated that gene expression was lost in most (27 of 34, 79%) of small cell lung carcinoma (SCLC) cell lines, but expression was retained in all 22 non-SCLC (NSCLC) lines tested. Loss of gene expression at the RNA level was associated with absent protein expression by Western blotting and lack of CASP8 enzymatic activity. Methylation of the promoter region of the CASP8 gene was present in 16 of 27 (59%) of the SCLC lines lacking gene expression. All methylated cell lines lacked the presence of an unmethylated allele indicating biallelic methylation or loss of non-methylated allele. Promoter methylation was absent in all SCLC and NSCLC cell lines retaining gene expression, and all of these lines had the unmethylated form of the gene. One non-expressing SCLC cell line, NCI-H82, had a homozygous deletion at 2q33 encompassing the chromosomal location of the CASP8 gene. The mechanism of gene inactivation in the remaining 10 of 27 (37%) non-expressing SCLC cell lines is unknown. Using five polymorphic markers for 2q33 a high frequency of allelic loss was present in SCLC lines. Analyses of fresh tumors showed that 15 of 43 (35%) of the SCLC, seven of 40 (18%) of bronchial carcinoids and none of 44 NSCLC tumors had CASP8 promoter methylation. Because only approximately 60% of SCLC cell lines lacking CASP8 expression were methylated, extrapolating from the cell line data, we estimate that approximately 58% of SCLC and 30% of bronchial carcinoids lack CASP8 expression. Thus, CASP8 expression is absent in a subset of both high grade (SCLC) and low grade (carcinoid) neuroendocrine lung tumors but not in NSCLC, which usually lack neuroendocrine features. CASP8 may function as a tumor suppressor gene in neuroendocrine lung tumors.

INTRODUCTION

Apoptosis or programmed cell death is a genetically regulated cellular suicide mechanism that plays a crucial role in embryogenesis, organ metamorphosis and tissue homeostasis.[1-3] While over 200 genes are known or suspected to be involved in apoptosis,[4] proteases called caspases (CASP) (cysteine aspartyl-specific proteases), which normally exist as latent zymogens, are key mediators of apoptosis when activated.[5] CASP8 plays a crucial role in the major apoptotic pathway involving death inducing ligands and their receptors, which are related to tumor necrosis factor (TNF) and its receptor.[6-7] These ligands include TNF, Fas ligand (FasL) and TRAIL. Binding of the death ligands to their specific receptors results in recruitment of the cofactor Fas associated death domain containing protein (FADD) with formation of a death inducing signaling complex (DISC) which results in activation of CASP8.8 Activation of the chief initiator caspase (CASP8) in turn activates the downstream executioner (effector) caspases, the key one being CASP3, which coordinate the execution phase of apoptosis by cleaving multiple structural and repair proteins.[9] The gene for CASP8 is located at chromosome 2q33, and a homozygous deletion involving this location has been identified in NCI-H82,[10] a SCLC cell line which we initiated.[11]

Aberrant methylation of normally unmethylated CpG islands located in the 5′ promoter region of genes has been associated with transcriptional inactivation of several genes in human cancer and can serve as an alternative to mutational inactivation.[12-18] A recent study reported that the gene for CASP8 is silenced preferentially in neuroblastomas with amplification of MYCN by a combination of promoter methylation and allelic deletion.[19] Thus CASP8 acts as a tumor suppressor gene in neuroblastomas. In this study, we investigated

inactivation of CASP8 in lung cancer cell lines and the methylation status of the gene in lung cancers.

MATERIALS AND METHODS

Cell Lines and Tumor and Tissue Samples. Because of the relative rarity of obtaining resected SCLC or bronchial carcinoids, tumor tissues were obtained from multiple sources. Resected NSCLC tumor tissues were predominantly from the USA, carcinoid tumors from France, and SCLC tumors from Japan. The NSCLC tumors and 32 of the SCLC tumors were from previously untreated patients, while 11 of the SCLC cases were from patients who had received prior cytotoxic therapy. The carcinoids tumors, all of which were from previously untreated patients, consisted of 33 typical and seven atypical carcinoids. All human lung cancer cell lines and corresponding B lymphoblastoid lines were initiated and characterized by us as described previously.[11,20]

Reverse Transcriptase-PCR (RT-PCR). Total RNA was extracted from cell lines using Triazol reagent (Life Technologies, Inc, Rockville, MD) following the manufacturer's instructions. The RNA isolated using this protocol was further purified to remove any traces of DNA using the DNA–free kit (Ambion, Austin, TX). RT was performed on total RNA using two rounds of PCR. In the first round first-strand cDNA synthesis was carried out using SUPERSCRIPT II reverse transcriptase (Life Technologies Inc, Rockville, MD) following manufacturer's protocol. In the second round PCR a specific sequence of the gene was amplified using Hot Star Taq DNA Polymerase (Qiagen Inc., Valencia, CA) as described before.[17] Forward and reverse primers are 5'-CAGCATTAGGGACAGGAATG-3'and5'-CAGTTATTCCACAGTGGCCAT-3' respectively.

Methylation Specific PCR (MSP). MSP was performed by bisulfite modification of genomic DNA as described by Herman et al,[21] PCR amplification of bisulfite modified DNA for CASP8 gene promoter was carried out using the methylated (M) and unmethylated (U) primers as described by Teitz et al.[19] Forward and reverse primers for the methylated sequences were 5'-TAGGGGATTCGGACATTGCGA-3'and 5'-GTATATCTACATTC-GAAACGA-3'. Forward and reverse primers for the unmethylated sequences were 5'-TAGGGGATTTGGAGATTGTGA-3'and 5'-CCATATATATC-TACATTCAAAACAA-3' respectively Artificially methylated DNA was used as a positive control in each PCR reaction along with water blank as negative control.

Western Blot Analysis. Cells were lysed in lysis buffer (1% TRITON-X, 20mM Na phosphate buffer pH 7.4, 150mM NaCl, 10% glycerol, 1mM phenylmethylsulphonyl fluoride). Samples normalized for total protein content (30 μg/lane) were separated by 10% SDS-PAGE, electroblotted on nitrocellulose and immunostained. Monoclonal antibody to CASP8 (BD Transduction Laboratories, San Diego, CA) was used according to the manufacturer's instructions. Detection of immunocomplex was performed using Supersignal (Pierce, Rockford, IL).

Aza-CdR or Trichostatin—A Treatment. Eight SCLC cell lines with CASP8 methylation were incubated in culture medium with and without the demethylating agent Aza-CdR at a concentration of 2 μg/ml for 7 days, with medium changes on days 1, 3, and 5. Cells were harvested at the end of the seventh day for extraction of total RNA, cDNA prepared by RT-PCR and tested for gene expression.

Eight SCLC cell lines lacking CSP8 expression (six methylated, two unmethylated) were treated with the histone deacetylase inhibitor trichostatin A (300 nM) and the cells harvested 24 hr later.[22]

Sequencing Analysis. PCR products were purified using ultrafree-MC Millipore column (Millipore, Bedford MA) and sequenced directly in both directions with Thermosequence dye terminator cycle sequencing pre-mix kits (ABI, Perkin-Elmer, Foster City, CA) and ABI 310S DNA Sequence System (Perkin-Elmer, Foster City, CA).

Loss of Heterozygosity (LOH) Analyses. Five microsatellite markers in 2q33 region flanking the CASP8 gene were selected for LOH analysis of DNA from paired SCLC and NSCLC tumor cell lines and their corresponding lymphoblastoid lines (as a source of constitutional DNA) and LOH analyses were conducted as described before.[23,24]

Enzymatic Assay for CASP8. The activity of CASP8 was determined using ApoAlert Caspase8 colorimetric assay kit, catalogue #K2029-1 (Clontech, Palo Alto, CA), following manufacturer's instructions. Briefly, CASP8 was activated in lung cancer cell lines with the treatment of human TNFα (Clontech, Palo Alto, CA). A control was set up for each cell line in which the culture was not induced. Cells were counted and centrifuged (3-5 x 10^6 cells at 400 x g for 10 min). The pellets were resuspended in 50 μL of chilled cell lysis buffer and incubated on ice for 10 minutes. The cell lysates were centrifuged in a microcentrifuge at 14,000 RPM for 3 min at 4°C to precipitate cellular debris. Fifty mL of 2x reaction buffer containing 10 mM DTT and 5 μL of the 4 mM IETD-pNA substrate were added to the supernatants and the reaction mixtures were incubated at 37°C for 2 hr in the dark. The release of pNA as the measure of CASP8 activity was read at 405 nm. The increase in Caspase-8 activity was determined by comparing the results with the uninduced controls. A calibration curve was generated using different concentrations of pNA (Sigma Chemical Co, St. Louis, MO). The slope of the curve was used to calculate the units of Caspase-8 activity.

Statistical Analyses. Statistical differences between groups were examined using Fisher's exact probability test. Values of $p < 0.05$ were considered significant.

RESULTS

Loss of Expression of CASP8 in Lung Cancer Cell Lines. In 1994 Yokota and coworkers identified a homozygous deletion at 2q33, the chromosomal location of CASP8, in the SCLC cell line, NCI-H82.[10] Because of this finding, we analyzed CASP8 in lung cancer cell lines using RT PCR. These data (Table 1, Fig. 1) demonstrated loss of CASP8 expression in 27 of 34 (79%) SCLC cell lines, including NCI-H82. By contrast, CASP8 expression was present in all 22 NSCLC lines (Table 1, Fig. 1). Further, Western blot analysis of these SCLC and NSCLC cell lines showed 100% concordance between protein expression and RNA expression (Table 1, Fig. 1).

CASP8 enzymatic activity was measured in lung cancer cell lines after TNFα stimulation in vitro. In seven SCLC lines lacking CASP8 expression by RT-PCR and western blotting we confirmed absence of the enzymatic activity of CASP8 expression following stimulation by TNFα g/ml) (Fig. 2). Two SCLC cell lines retaining expression had varying levels of CASP8 activity. By contrast, all four NSCLC cell lines tested (all of which were positive for CASP8 expression) had, in general, higher levels of activity.

DNA Methylation Status of CASP8. To test for methylation of the promoter region of CASP8, we used a CpG-rich region of the 5' flanking region for designing oligonucleotide primers for methylation-sensitive PCR analysis as described before by Teitz et al.[19] Using the CASP8 methylation specific primers and bisulfite-treated genomic DNA from lung cell lines as a substrate, a 321bp PCR product was detected in 16 of the 27 (59%) SCLC cell lines lacking expression of CASP8 (Table 1, Fig. 1). DNA sequencing of the 321bp PCR product, further confirmed that CpG sites in the putative CASP8 promoter sequences of all these 16 SCLC samples were methylated. Using CASP8 unmethylated primers and bisulfite-treated genomic DNA from lung cell lines as a substrate a 322bp PCR product was detected in all the 22 NSCLC and 7 SCLC cell lines that showed expression of CASP8 (Table 1, Fig. 1). In addition, two SCLC that were negative for CASP8 expression had both the methylated and unmethylated bands. As expected the homozygously deleted cell line NCI-H82 lacked both forms. Of the remaining SCLC and NSCLC cell lines, only the methylated or unmethylated forms of the gene were present. These results confirm the homozygous deletion in NCI-H82, and indicate loss of the unmethylated allele in methylated cell lines. Direct sequencing of methylated DNA PCR products from all 16 methylation positive SCLC cell lines confirmed that all cytosines not at CpG sites were converted to uracils by bisulfite treatment, whereas the cytosines at all three CpG sites remained unchanged.

We treated eight methylated SCLC cell lines with the demethylating agent Aza-CdR. After 7 days of treatment, gene expression could be detected in six of the eight cell lines by RT-PCR. (Fig. 3), concomitant with the hypothesis that methylation was responsible for gene silencing in these lines.

Table 1 **Analysis of CASP8 Expression and DNA Methylation in SCLC and NSCLC Cell Lines[a]**

Cell Lines[a]	Western Blotting +ve	Methylated Form	Unmethylated Form
SCLC			
All (n=34)	7 (21%)	16 (47%)	19 (56%)
RT PCR +ve (n=7)	7 (100%)	0 (0%)	7 (100%)
RT PCR –ve (n=27)	0 (0%)	16 (59%)b	12 (44%)b
NSCLC			
All (n=22)	22 (100%)	0 (0%)	22 (100%)
RT PCR +ve (n=22)	22 (100%)	0 (0%)	22 (100%)
RT PCR –ve (n=0)	0 (0%)	0 (0%)	0 (0%)

[a]P RT CR, Western Blot and Methylation analyses of caspase 8 were carried out as described in Materials and Methods; bIncludes two lines having both methylated and unmethylated forms and one line(NCI-H82) lacking both forms.

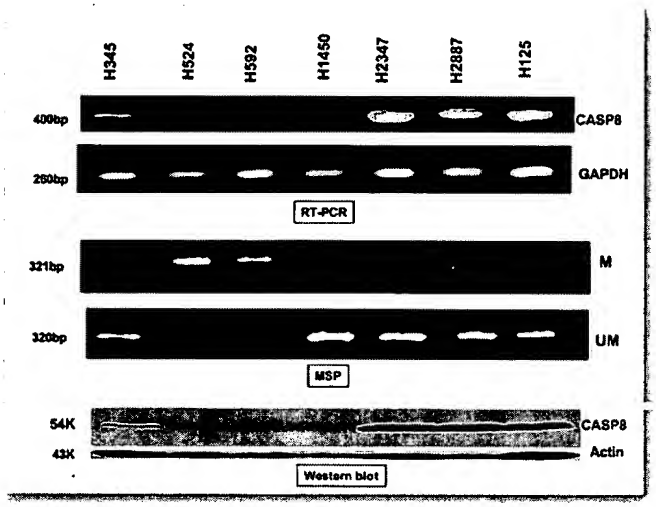

Figure 1. MSP and expression analysis of the CASP8 gene in lung cancer cell lines. All cell lines are preceded by the prefix NCI-. Cell lines H345, H524, H592, and H1450 are SCLC while H2347, H2887 and H125 are NSCLC. A. RT-PCR expression analysis of cDNA of CASP8 gene in lung cancer cell lines by RT-PCR analysis. GAPDH was used as a control for RNA integrity. H524, H592 and H1450 represent SCLC cell lines which were negative for CASP8 expression while H345 is a SCLC cell line which was positive for CASP8 expression. B. MSP analysis of CASP8. The 321bp of PCR product corresponding to the methylated allele (cell lines H524, H592) and the 322bp of PCR product corresponding to the unmethylated allele (cell lines H345, H1450, H2347, H2887 and H125) are shown. The methylated primers detect only DNA that contain methylated CpG dinucleotides. C. Expression of CASP8 gene in lung cancer cell lines by western blot (WB). 30mg of total protein was used per lane. B-actin was used to normalize the amount protein in each sample.

Treatment with trichostatin A of methylated or unmethylated SCLC cell lines lacking CSP8 expression failed to induce gene expression.

LOH Analysis of Lung Cancer Cell Lines. Of the 56 lung cancer cell lines studied for CASP8 expression and methylation status, paired B-lymphoblastoid cell lines were available from 30, and were utilized as sources of constitutional DNA. These 30 lines were tested for LOH at 2q33 using five polymorphic markers (Fig. 4). LOH involving one or more markers was present in seven of 13 (53%) of SCLC cell lines and in five of 17 (29%) NSCLC lines. The CASP8 gene is reported to be located between markers D2S2392 and D2S307.[25] Of interest, allelic loss at D2S2392 was present in four of six (67%) informative SCLC lines and in three of 10 (30%) of informative NSCLC lines. Allelic loss at D2S307 was present in four of five (80%) informative SCLC lines and in one of six (17%) informative NSCLC lines. While, these differences did not reach statistical significance, due to the small sample size, we have previously demonstrated that allelic losses of less than 30% probably reflect background genomic instability.[20]

Methylation of CASP8 in Neuroendocrine Lung Tumors. We confirmed and extended the cell line observations by studying methylation of CASP8 promoter region in frozen lung tumors. Of the 43 SCLC analyzed 15 (35%) and none of 44 NSCLC tumors were positive for methylation (Table 2). Additionally, of the 40 carcinoids analyzed 7 (18%) were positive for methylation. Twelve of the methylated SCLC and all of the methylated carcinoids were from previously untreated patients while three of the methylated SCLC tumors were from patients who had received cytotoxic therapy.

DISCUSSION

Evasion of apoptosis has been described as one of the six hallmarks of cancer.[26] Tumor cells are under tremendous pressure to undergo apoptotic death from both internal and external sources, mandating effective mechanisms of defense for survival. Paradoxically, SCLC tumors and cell lines spontaneously exhibit both high proliferation and apoptotic rates.[27,28] Alterations in the ratios of the proapoptotic and antiapoptotic members of the *Bcl-2* family members have been described in lung cancers.[29,30] Inactivation of the DAP kinase gene by methylation has been reported to be associated with advanced stage in NSCLC.[31] In a small series of cell lines, Joseph et al, demonstrated differential loss of expression of caspases 8, 10, 1 and 4 in SCLC.[32]

Recent studies have shown that methylation of the promoter region of CASP8 combined with allelic loss at 2q33 are the major mechanisms for gene inactivation in neuroblastomas.[19,33,34] A prior report that SCLC cell line NCI-H82 had a homozygous deletion at 2q33,[10] the chromosomal location of the CASP8 gene, led us to investigate the status of the gene in this cell line. We found that the homozygous deletion in NCI-H82 encompassed the CASP8 gene, prompting us to study the status of the gene in lung carcinoma cell lines and tumors. By RT-PCR we found frequent loss of CASP8 expression (78%) in SCLC cell lines but not in any NSCLC line tested. This is one of the few absolute molecular differences between SCLC and NSCLC described to date.

Because aberrant methylation of promoter regions is the major mechanism of tumor suppressor gene silencing in cancers, we used the MSP method to investigate the methylation status of the promoter region of the CASP8 gene. The region examined for methylation by us and by others is 300 bp downstream from TATA box and 200 bp downstream from the transcription initiation site and is located within the 5′ untranslated region of the gene. The amplicon, consisting of 320 bp, contains 6 CpG sites, has a C + G content of 51% and an observed to expected CpG ratio of 0.3. Thus it only partially satisfies the criteria of a CpG island.[35] Despite this finding, there is strong supportive evidence that methylation (but not histone deacetylation) may play a role in CASP8 gene silencing:

1. Methylation was present in 62% of SCLC cell lines that did not express CASP8 (excluding a cell line with homozygous deletion);
2. methylation was absent in 29 lung cancer cell lines that retained gene expression;
3. treatment with the demethylating agent Aza-CdR restored CASP8 expression in 75% of SCLC cell lines tested; and

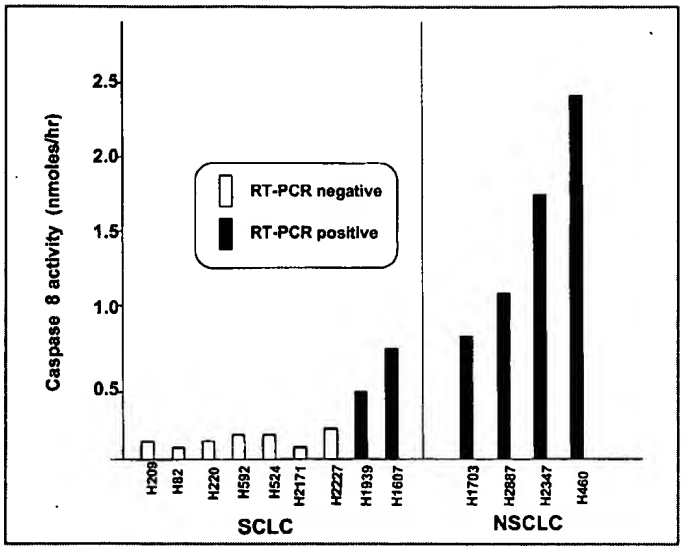

Figure 2. Enzymatic activity of CASP8 after TNFα induction of lung cancer cell lines, determined using ApoAlert Caspase-8 colorimetric assay kit. SCLC cell lines (H209, H82, H220, H592, H524, H2171, H2227) which were negative for CASP8 expression showed very low, presumably background CASP8 activity. SCLC cell lines (H1939, H1607) which were positive for CASP8 expression showed intermediate levels and NSCLC cell lines (H1703, H2887,H-2347 and H460) which were positive for CASP8 expression showed higher CASP8 activity.

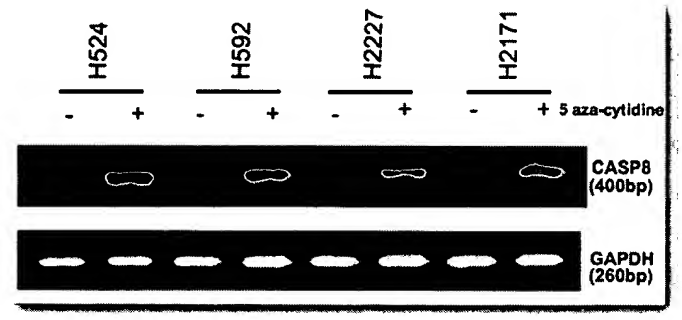

Figure 3. Re-expression of CSP8 by RT-PCR after after Aza-CdR treatment. Figure illustrates examples of four expression negative cell lines before and after treatment with Aza-CdR (AzaC). GAPDH 260 bp RT-PCR shown as a cDNA control.

4. The histone deacetylase inhibitor trichostatin A did not restore expression in any of six cell lines tested.

In addition, with rare exceptions, cell lines with a methylated allele lacked the corresponding unmethylated allele, indicating either biallelic methylation or allelic loss of the unmethylated allele. As expected, the homozygously deleted cell line NCI-H82 lacked both forms. However, we are unable to account for the mechanism of inactivation of the gene in 37% of SCLC cell lines. Of interest, some genes such as p16 are inactivated in lung cancers by multiple mechanisms.[36] In addition, rare missense mutations of CASP8 have been described in neuroblastomas.[34]

Using polymorphic markers, we demonstrated a higher frequency of allelic loss at 2q33in SCLC cell lines than in NSCLC lines. Previously, in screening studies that did not target this particular chromosomal location, we failed to find frequent losses at 2q33 in lung cancer cell lines.[20,37] In particular, losses in SCLC lines at the two markers spanning the gene location, D2S2392 and D2S307

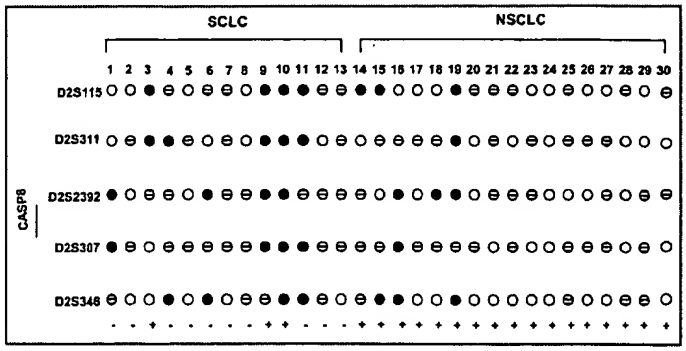

Figure 4. Schematic representation of LOH on chromosome 2q in SCLC and NSCLC compared to paired B lymphoblastoid cell lines.. Closed circle—LOH; open circle—retention of heterozygosity; striped circle—non-informative. The order of markers and location of CASP8 gene are as shown on the left. The distance between the two markers flanking the CASP8gene is estimated to be approx.1.5 cM. Plus and minus signs at the bottom of the figure represent presence or absence of CASP8 expression respectively in that particular cell line.

were much higher (67-80%) in SCLC lines than in NSCLC lines (17-30%). We have previously demonstrated that losses of individual markers in lung cancer cell lines above 30% were abnormal and may indicate the location of tumor suppressor genes, while losses less than 30% probably represented "background" losses from genomic instability.[20] In the small number of informative SCLC cell lines analyzed, significant correlations between allelic loss and gene expression or methylation could not be demonstrated. It has been suggested that one or more other tumor suppressor genes are located within the 2q33 region and PLC-L has been suggested as a candidate.[38]

In both SCLC and NSCLC cell lines, we found a 100% concordance between gene expression by RT-PCR and protein expression by Western blotting. In a subset of cell lines, gene expression of CSP8 correlated with functional activity. Of interest we have recently demonstrated that caspase 10 protein expression may be absent in lung and breast cancer cell lines even though RT-PCR expression was present.[39]

We confirmed our methylation findings by testing tumor DNA derived from lung cancers. Because SCLC tumors are only occasionally resected and carcinoids are relatively rare tumors, only DNA was available from them. Of interest, methylation was found in 35% of SCLC and in 18% of carcinoids. Because only approximately 60% of SCLC cell lines lacking CASP8 expression were methylated, extrapolating from the cell line data, we estimate that approximately 58% of SCLC and 30% of bronchial carcinoids lack CASP8 expression.

Both SCLC and carcinoids are neuroendocrine tumors, while NSCLC tumors usually lack these features.[40,41] However, bronchial carcinoids are not smoking associated and have a low malignant/metastatic potential, while the heavily smoking associated SCLC tumors are widely metastatic. In addition, mutations of the MEN1 gene are frequent in carcinoids and absent in SCLC.[42,43] Thus, CASP8 methylation is probably related to neuroendocrine differentiation rather than to the very different pathogenetic origins of these two tumor types. Of interest, neuroblastoma, the only other tumor type in which CASP8 loss has been demonstrated, is also a neuroendocrine tumor.

Our findings indicate frequent inactivation of the important apoptosis associated CASP8 gene in neuroendoendocrine tumors of

Table 2 **Frequency of CASP8 Methylation in Lung Tumors and Lung Cancer Cell Lines[a]**

	CSP8 Methylation	
	Tumors	Cell lines
SCLC	15/43 (35%)[b]	16/34 (47%)[b]
Carcinoids	7/40 (18%)	NA
NSCLC	0/44 (0%)[b]	0/22 (0%)[b]

[a]Methylation analysis of CSP8 was performed as described in Materials and Methods; [b]Differences between SCLC group and NSCLC groups were statistically significant ($p<0.05$).

the lung. Our data suggests that the mechanism of inactivation frequently but not always involves a combination of methylation and allelic deletion. Of interest, our collaborators have described frequent polymorphisms in NSCLC of the ectodomain of DR4,[44] a receptor for the death inducing ligand TRAIL, as well as loss of CASP10 in lung cancers.[39] Thus the death ligand-receptor pathway to apoptosis may be disrupted in both neuroendocrine and non-neuroendocrine tumors of the lung by multiple mechanisms.

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Deregulation of *Caspase 8* and *10* Expression in Pediatric Tumors and Cell Lines[1]

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ABSTRACT

Methylation of the promoter regions of CpG-rich sites in genes is the major mechanism for the silencing of many genes in tumors. Methylation of the key apoptosis-related gene *caspase 8* (*CASP8*) has been reported in some childhood tumors and in neuroendocrine lung tumors. We examined the methylation status of 181 pediatric tumors and found frequent methylation in rhabdomyosarcomas (83%), medulloblastomas (81%), retinoblastomas (59%), and neuroblastomas (52%). Methylation frequencies were low in Wilms' tumors (19%) and absent in hepatoblastomas, acute leukemias, osteosarcomas, Ewing's sarcomas, and ganglioneuromas and in normal tissues. Methylation of *CASP8* and the tumor suppressor gene *RASSF1A* were highly significantly correlated in all tumor types by both the χ^2 and the Fisher's exact tests ($P < 0.0001$ for both tests). Because the region of the gene examined by us and others is not located in the promoter region and lacks features of a CpG island, we explored the relationship between methylation and gene silencing in detail using 23 pediatric tumor cell lines. Studies included relating the methylation of the region to gene expression at mRNA and protein levels, enzymatic assays of gene function, clonal analysis of PCR amplicons of the region, and exposure to a demethylating agent. These studies indicated that methylation correlated with the loss of gene function in most cases; however, other mechanisms of gene inactivation were present in some cases. Posttranscriptional inactivation of the closely related gene *caspase 10* was present in many cell lines. Our results suggest that deregulation of the death receptor pathway to apoptosis is frequent in many types of pediatric tumors and their cell lines.

INTRODUCTION

The caspases, including *CASP8*[3] and *CASP10*, are cysteine proteases involved in apoptosis. *CASP8* and *CASP10* are upstream or initiator caspases, involving death receptors and their ligands such as TRAIL and TNF. Binding of death receptors to their ligands results in recruitment and activation of *CASP8* and *CASP10*, formation of the death-inducing signaling complex (DISC) and subsequent initiation of the apoptotic cascade (1). *CASP8* may play an essential role in apoptosis induced by chemotherapeutic agents and irradiation (2, 3). The role of the closely related gene *CASP10*, located in the same gene complex at 2q33 (4), is more controversial. Our recent studies indicate that each caspase can initiate apoptosis independently of the other (5). A novel molecule called *c-FLIP* (FADD like interleukin-1 converting enzyme inhibitory protein) located at 2q33, resembles *CASP8* in overall structure but is proteolytically inactive (6). In contrast to *CASP8* and *10*, overexpression of *c-FLIP* has been shown to protect against apoptosis mediated by FasL and TRAIL in several cancer cell lines *in vitro* (6).

Recent studies have reported that *CASP8* was frequently inactivated by a combination of methylation and allelic deletion at 2q33 (the chromosomal location of the gene) in neuroblastomas with *MYCN* amplification (7–10). *CASP8* was methylated with a loss of gene expression in 55% of medulloblastomas (11). Except for a small number of Ewing's sarcoma and rhabdomyosarcoma cell lines (which retained expression and lacked methylation; Refs. 9, 12), other pediatric tumors have not been examined. We have recently demonstrated that *CASP8* is methylated and inactivated in neuroendocrine lung carcinomas (small cell and bronchial carcinoids) but not in non-small cell carcinomas (13). In addition, loss of *CASP10* protein expression may occur in breast and lung carcinomas that retain message expression (5).

In this report, we examined the methylation status of the gene in 181 tumors representing the major forms of pediatric tumors. In addition, we investigated expression (at the message and protein level) and *CASP8* activity and their relationship to methylation of the gene and expression of *CASP10* and *c-FLIP* in pediatric tumor cell lines.

MATERIALS AND METHODS

Tumor Samples and Cell Lines. A total of 181 and 23 pediatric tumors and cell lines were examined. Most of the tumor samples ($n = 144$) were obtained from Children's Hospital Medical Center, Dallas, TX, after obtaining Institutional Review Board approval and informed consent. Twenty hepatoblastoma samples were obtained from the Pediatric Oncology Group Hepatoblastoma Tumor Bank. The retinoblastomas ($n = 17$) were from the University of Siena, Siena, Italy. Rhabdomyosarcoma samples included alveolar ($n = 6$), embryonal ($n = 7$), and anaplastic types ($n = 2$). Eighteen acute lymphoblastic (ALL) and two acute myelogenous leukemia (AML) samples were included in the acute leukemia cases. Nonmalignant samples included corresponding histologically normal kidney ($n = 11$) from Wilms' tumor cases and liver ($n = 1$) from a hepatoblastoma case, peripheral blood lymphocytes ($n = 2$) from healthy adults, and autopsy tissues from patients without cancer (six muscle, six lung, and five liver samples; Tables 1 and 2).

Twenty-three pediatric tumor cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA; Table 1). Cell lines were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with the optimal concentration (10–20%) of fetal bovine serum and incubated in 5% CO_2 at 37°C.

MSP Assay. Genomic DNA was isolated from frozen tissues and cell lines by SDS and proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation (14). DNA was treated with sodium bisulfite as described previously (15). Two μl of bisulfite-modified DNA was amplified by PCR, using primers that were specific for methylated or unmethylated sequences of *CASP8* gene as described previously (7). PCR-amplified products were electrophoresed on 2% agarose gel and visualized under UV illumination.

RT-PCR. Total RNA was extracted from cell lines using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufactur-

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[3] The abbreviations used are: CASP8, caspase 8; CASP10, caspase 10; TRAIL, TNF-related apoptosis-inducing ligand; c-FLIP, cellular FLICE inhibitory protein; RASSF1A, Ras association domain family 1, isoform A; TNF, tumor necrosis factor; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR; 5-aza-CdR, 5-aza-2′ deoxycytidine; TSA, tricostatin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IETD-pNA, isoleucine glutamic acid threonine aspartic acid tetrapeptide paranitroamiline.

Table 1 *Methylation status of CASP8 and expression of mRNA in pediatric tumor cell lines*

Designation	ATCC no.[a]	Tumor type	M	UM	mRNA
SK-N-AS	CRL-2137	Neuroblastoma	−	+	+
BE(2)-C	CRL-2268	Neuroblastoma	−	+	+
CHP-212	CRL-2273	Neuroblastoma	−	+	+
SK-N-MC	HTB-10	Neuroblastoma	−	+	+
A-673	CRL-1598	Rhabdomyosarcoma	−	+	+
SJRH30	CRL-2061	Rhabdomyosarcoma	−	+	+
A-204	HTB-82	Rhabdomyosarcoma	−	+	+
G-401	CRL-1441	Rhabdomyosarcoma	−	+	+
DAOY	HTB-186	Medulloblastoma	−	+	+
SAOS-2	HTB-85	Osteosarcoma	−	+	+
IMR-32	CCL-127	Neuroblastoma	+	−	−
SH-SY5Y	CRL-2266	Neuroblastoma	+	−	−
BE(2)-M17	CRL-2267	Neuroblastoma	+	−	−
SK-N-BE(2)	CRL-2271	Neuroblastoma	+	−	−
D283	HTB-185	Medulloblastoma	+	−	−
D341 MED	HTB-187	Medulloblastoma	+	−	−
Y-79	HTB-18	Retinoblastoma	+	−	−
WERI-RB-1	HTB-169	Retinoblastoma	+	−	−
SK-N-FI	CRL-2142	Neuroblastoma	+	+	+
SK-N-SH	HTB-11	Neuroblastoma	+	+	+
RD	CCL-136	Rhabdomyosarcoma	+	+	+
SK-NEP-1	HTB-48	Wilms' tumor	+	+	+
SK-N-DZ	CRL-2149	Neuroblastoma	+	+	−

[a] ATCC no., the American Type Culture Collection number; M, methylated form; UM, unmethylated form; +, a band was detected; −, a band was not detected.

er's instructions. Two μg of total RNA was reverse-transcripted by use of SUPERSCRIPTII First-Strand synthesis (Invitrogen, Carlsbad, CA), and then 1 μl of cDNA was used for the amplification of mRNA of *CASP8* and *CASP10*. Because multiple isoforms of *CASP8* have been described, we used two sets of primers for its expression. One was as described previously (7) for exons 8 and 9, and the other set was designed by us for exons 1 and 3: sense, 5′-GGGAAGTGTTTTCACAGGTT-3′ and antisense, 5′-TTCTTGCTTC-CTTTGCGGAAT-3′. Primer sequences for *CASP10* were: sense, 5′-AA-GAAACAGATGCCCCAGCCTG-3′ and antisense, 5′-CGAGACTACAGT-GAGCCGTGATTG-3′. *GAPDH*, a housekeeping gene, was used as a control for RNA integrity, as described previously (16). PCR products were electrophoresed on 2% agarose gel and visualized under UV illumination.

5-aza-CdR and TSA Treatment. Cell lines lacking gene expression were treated with 5-aza-CdR at a concentration of 0.5–2 μg/ml for 5 days. TSA treatment was performed at a concentration of 300 nM for 24 h. Duplicate flasks before and after treatment were harvested and tested for gene expression.

Western Blot Analysis. Cells were placed in lysis buffer [0.5% NP40, 10 mM Tris HCl (pH 8.0), 150 mM NaCl, 3 mM $MgCl_2$, and 2 mM phenylmethylsulphonyl fluoride]. Samples normalized for total protein content (30 μg/lane) were separated by 10% SDS-PAGE, electroblotted on nitrocellulose, and immunostained. Monoclonal antibodies to *CASP8* (Oncogene, Boston, MA) and *CASP10* (MBL International Corporation, Watertown, MA) were used according to the manufacturers' instructions. A rabbit monoclonal antibody to *c-FLIP* was generated by one of us (P. M. C.). Detection of immunocomplex was performed using Supersignal (Pierce, Rockford, IL).

***CASP8* Activity Assay.** The activity of *CASP8* was determined using ApoAlert Caspase8 colorimetric assay kit (Clontech, Palo Alto, CA) by following the manufacturer's instructions. Briefly, *CASP8* was activated in pediatric tumor cell lines by the treatment of 100 ng/ml human TNF-α (Clontech) for 24 h. Untreated cells were used as a control for each cell line. Cells were counted (3–5 × 10^6 cells) and centrifuged (3,000 rpm for 10 min). The pellets were resuspended in 50 μl of cell lysis buffer and incubated on ice for 10 min, and cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Fifty μl of 2× reaction buffer containing 10 mM DTT and 5 μl of 4 mM IETD-pNA substrate were added to the supernatants, and the reaction mixtures were incubated at 37°C for 2 h in the dark. The release of pNA as the measure of *CASP8* activity was read at 405 nm. The increase of *CASP8* activity was determined by comparing the results with untreated controls. A calibration curve was generated using different concentrations of pNA (Sigma, St. Louis, MO). The slope of the curve was used to calculate the units of *CASP8* activity.

Bisulfite-modified Sequencing Analysis. Bisulfite-modified DNA was amplified by PCR using two sets of primer in the *CASP8* gene. Primer sequences were: for CASP8SQ1; sense, 5′-TGTAAGAAAGAATGG-TATATTA-3′, and antisense, 5′-ATAAAAACACTTCCCTCCAAC-3′; and for CASP8SQ2, sense, 5′-AGAGTTAGGGTGGTTATTGA-3′, and antisense, 5′-AATAAAATCTTCTAAACTCTCC-3′. The PCR products were cloned into pCR2.1-TOPO vector (Invitrogen); 10 clones from each of the samples were selected, and plasmid DNAs were purified using QIAprep Spin Miniprep kits (Qiagen, Valencia, CA). DNA sequencing was performed by ABI PRISM 377 (Applied Biosystems, Foster City, CA) to determine the methylation status of *CASP8*.

Statistical Analysis. Statistical analyses for differences between groups were performed using the χ^2 and the Fisher's exact tests and the nonparametric Mann-Whitney *t* test. Kaplan-Meier log-rank test was performed to calculate overall survival.

RESULTS

Methylation of *CASP8* in Pediatric Tumors and Cell Lines. Methylation of *CASP8* was present in 58 (32%) of 181 pediatric tumors and 13 (57%) of 23 cell lines (Tables 1 and 2; Figs. 1 and 2*A*). Rhabdomyosarcomas (83%), medulloblastomas (81%), retinoblastomas (59%) and neuroblastomas (52%) were frequently methylated. However, the rate of methylation of Wilms' tumors was 19%, and methylation was absent in hepatoblastomas, acute leukemias, osteosarcomas, and Ewing's sarcomas. *CASP8* methylation was present in the three major subtypes of rhabdomyosarcomas (4 of 6 alveolar, 9 of 10 embryonal, and 2 of 2 anaplastic types). In contrast to neuroblastomas, the differentiated, much-less-aggressive ganglioneuromas ($n = 6$) lacked *CASP8* methylation. Despite the high rate of *CASP8* methylation in primary rhabdomyosarcoma samples (83%), only one of five cell lines (20%) was methylated. Methylation was absent in all of the normal samples examined.

Methylation Status of *CASP8* and Clinicopathological Parameters. We examined the relationship between methylation of *CASP8* and clinicopathological parameters including sex, age, stage, existence of metastasis, and outcome. Neuroblastoma patients with methylation of *CASP8* were significantly older than patients whose tumors lacked methylation (2.31 ± 1.37 years old *versus* 0.87 ± 1.40 years old, respectively; $P = 0.002$). There was no relationship between methylation status and other clinicopathological parameters.

Table 2 *Frequencies of methylation of CASP8 in pediatric tumors and tumor cell lines*[a]

		n (%)
Total	T = 181	58 (32)
	C = 23	13 (57)
Rhabdomyosarcoma	T = 18	15 (83)
	C = 5	1
Medulloblastoma	T = 16	13 (81)
	C = 3	2
Retinoblastoma	T = 17	10 (59)
	C = 2	2
Neuroblastoma	T = 27	14 (52)
	C = 11	7 (64)
Ganglioneuroma	T = 6	0
Wilms' tumor	T = 31	6 (19)
	C = 1	1
Hepatoblastoma	T = 27	0
Acute leukemia	T = 20	0
Osteosarcoma	T = 11	0
	C = 1	0
Ewing's sarcoma	T = 8	0
Normal samples	*n* = 14	0

[a] T, primary tumor; C, cell line.

Fig. 1. MSP of *CASP8* in representative samples of pediatric tumors. The results of methylated forms of *CASP8* are illustrated. *WT*, Wilms' tumor; *NB*, neuroblastoma; *HB*, hepatoblastoma; *RMS*, rhabdomyosarcoma; *MB*, medulloblastoma; *OS*, osteosarcoma; *EWS*, Ewing's sarcoma; *RB*, retinoblastoma; *AL*, acute leukemia; *NT*, normal samples; *M*, size marker; *P*, positive control (lung cancer cell line previously found to be methylated); *N*, negative control (water blank). The unmethylated form of $p16^{INK4A}$ was used as a control for DNA integrity and was present in all of the samples (data not shown).

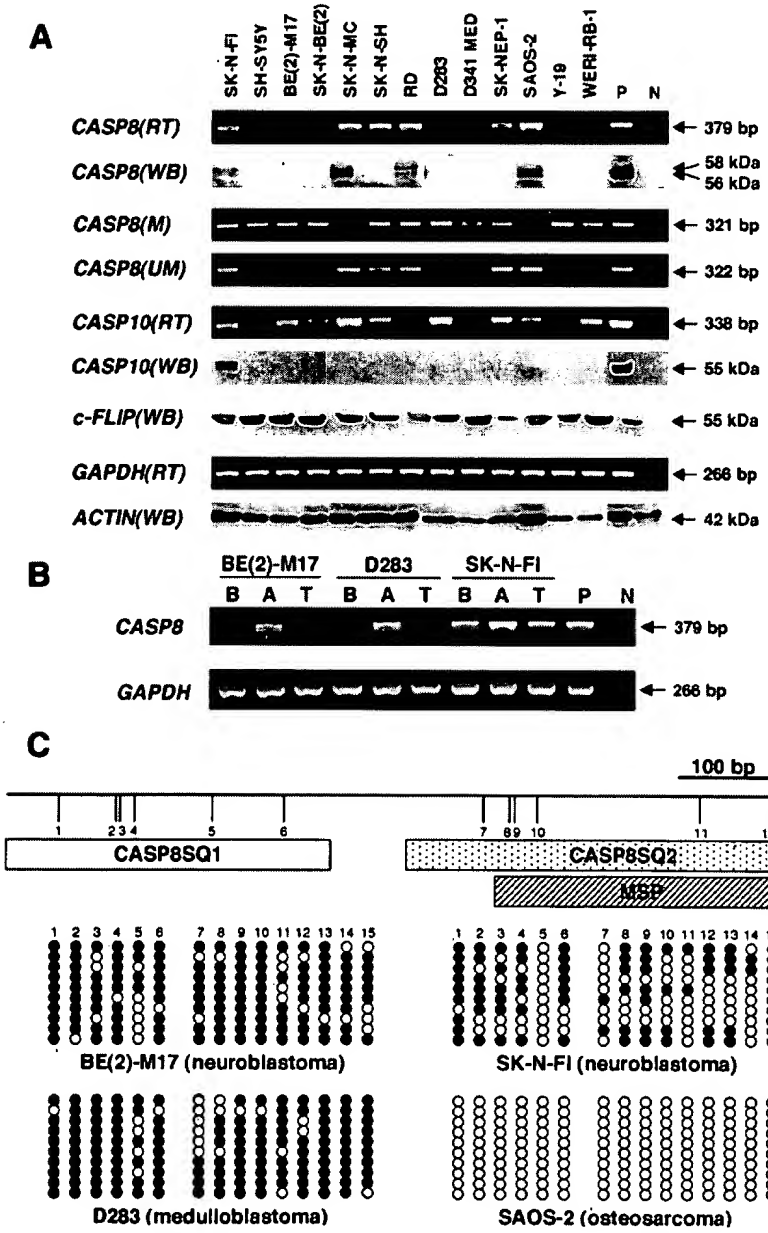

Fig. 2. State of *CASP8*, *CASP10*, and *c-FLIP* genes in pediatric tumor cell lines. *A*, methylation status of *CASP8* (by MSP assay) and expression of *CASP8*, *CASP10*, and *c-FLIP* in representative pediatric tumor cell lines. The results of 13 cell lines are illustrated. Expression of mRNA of *CASP8* and *CASP10* was examined using RT-PCR (*RT*), and protein expression of all three genes by Western blot analysis (*WB*). GAPDH and actin were used as internal controls for RT-PCR and Western blot analyses, respectively. *M*, methylated allele; *UM*, unmethylated allele; *P*, positive control; *N*, negative control (water blank for MSP and RT-PCR assays; lysates of cell lines previously found to be negative for Western blot analyses). *B*, expression of mRNA of *CASP8* in representative pediatric tumor cell lines before and after treatment with 5-aza-CdR and TSA by RT-PCR. Cell lines BE(2)-M17 and D283 had only a methylated allele and lacked an unmethylated allele. SK-N-FI had both alleles (see part *A* above). Lines BE(2)-M17 and D283 lacked expression of *CASP8* before treatment (*B*), but its expression was restored only after 5-aza-CdR (*A*) but not TSA (*T*) treatment. In SK-N-FI, expression of *CASP8* was present before treatment, and its expression increased after 5-aza-CdR treatment but not after TSA treatment. *P*, positive control; *N*, negative control (water blank); *kDa*, M_r in thousands. *C*, bisulfite-modified sequencing analysis of the *CASP8* gene in pediatric tumor cell lines. The four cell lines were examined and their methylation status and expression of *CASP8* by MSP and RT-PCR are shown (see part *A* above). The *top panel* illustrates the region examined including parts of exon 1 and intron 1 of *CASP8* (GenBank accession no. AF210257, nucleotides 32–900; AC007256, nucleotides 9716–10584 respectively) *vertical bars*, CpG sites. *Three rectangular boxes*, the amplicons resulting from sequence analysis (*CASP8SQ1*, *CASP8SQ2*) and for MSP assay (*MSP*). *Bottom panel* illustrates the methylation status of individual CpG sites in clones of the four cell lines. ●, methylated CpG sites; ○, unmethylated CpG sites. In BE(2)-M17 and D283, the CpG sites were extensively methylated; in SAOS-2, they were completely unmethylated. In these three cell lines, methylation and gene expression show negative concordance. In SK-N-FI, heterogeneity was noted, in keeping with the presence of both methylated and unmethylated alleles and with gene expression.

Relationship between *CASP8* and *RASSF1A* Methylation or N-*MYC* Amplification. We previously reported that the *RASSF1A* gene, a newly described tumor suppressor gene located at 3p21.3 (17), was frequently methylated in pediatric tumors (18). Therefore, we examined the relationship between *CASP8* and *RASSF1A* methylation. There was a significant relationship between them in all of the pediatric tumors ($P < 0.0001$), in retinoblastoma ($P < 0.0001$), and in neuroblastoma ($P = 0.0039$). Amplification of the N-*MYC* gene in neuroblastoma is a negative prognostic factor and has been associated with *CASP8* methylation (8). As a requirement for enrollment onto Pediatric Oncology Group clinical protocols, the patients with neuroblastic tumors had the status of the N-*MYC* gene in their tumor tissues examined by St. Jude Children's Research Hospital, Memphis TN. Most of the cases were early stage and only two of the neuroblastomas and ganglioneuromas were amplified for N-*MYC* by fluorescence *in situ* hybridization analysis (*i.e.*, copy number >10), and neither of these cases was methylated for *CASP8*.

Expression of mRNA and Protein of *CASP8* in Cell Lines. We examined expression of *CASP8* mRNA by RT-PCR in 23 pediatric tumor cell lines (Table 1; Figs. 2*A* and 3). Expression was present in 14 cell lines (61%). Of these, 10 expressed only the unmethylated alleles, whereas 4 cell lines had both methylated and unmethylated alleles. Expression was absent in nine (39%) cell lines. These cell lines had only methylated alleles and lacked unmethylated alleles of *CASP8* except for one neuroblastoma line, SK-N-DZ, which had both alleles. We also performed Western blot analysis for *CASP8* in 13 cell lines. We detected the two isoforms, caspase-8a and -8b (58,000 and 56,000 daltons, respectively) as described previously (19). There was good concordance between RT-PCR and protein expression, although cell lines SK-N-SH and SK-NEP-1 expressed mRNA but no protein.

We also performed an assay for *CASP8* activity after TNF-α stimulation in 13 cell lines (Fig. 3). Although *CASP8* activity was variable, it was concordant with protein expression in 11 cell lines. Cell lines WERI-RB-1 and D341 MED had no detectable protein expression but had weak activity.

5-aza-CdR and TSA Treatment. To determine whether methylation of *CASP8* is associated with transcriptional silencing, we examined expression of *CASP8* mRNA before and after treatment with 5-aza-CdR, a demethylating agent, by RT-PCR in 11 cell lines (Fig. 2*B*). We found restoration of *CASP8* mRNA after 5-aza-CdR treatment in six (SH-SY5Y, BE(2)-M17, SK-N-BE(2), D283, Y-79, WERI-RB-1) of seven methylated cell lines but not in D341 MED. Four cell lines (SK-N-FI, SK-N-SH, RD, and SK-NEP-1), which had both alleles and expression, were also treated with 5-aza-CdR. An increase of expression of *CASP8* mRNA was detected in three of these four cell lines except for SK-N-SH. Restoration of *CASP8* mRNA was not detected in two cell lines (D341 MED, SK-N-SH).

We also treated the 11 cell lines with TSA, an inhibitor of histone deacetylase activity. TSA treatment failed to restore expression in the seven methylated cell lines lacking expression of *CASP8*. In only one cell line, RD, having both methylated and unmethylated alleles and expression, did the intensity of mRNA expression increase slightly after treatment.

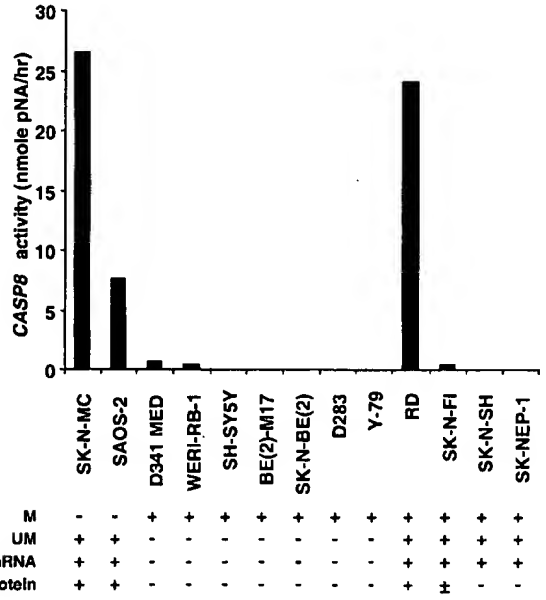

Fig. 3. *CASP8* activity after TNF-α treatment in pediatric tumor cell lines using ApoAlert Caspase8 colorimetric assay kit. *Lower columns*, characteristics of the cell lines tested: methylation status by MSP (*M*, methylated allele; *UM*, unmethylated allele), mRNA expression by RT-PCR (*mRNA*), and protein expression by Western blot (*Protein*). +, positive; ±, weak positive; −, negative.

Sequencing of *CASP8* after Bisulfite Modification. In the original report on methylation of *CASP8* in neuroblastomas, Teitz *et al.* (7) reported that they examined the 5′ flanking region of the gene, presumably the site of the promoter region. However, according to sequencing data of *CASP8* (GenBank accession no. AF210257), the region examined is downstream from the TATA box and is located within the 5′ untranslated region of the gene. As we have discussed previously (13), this region only partially satisfies the criteria of a CpG island (20), and the total number of CpG sites (seven) within the MSP amplicon is relatively small. Despite these findings, we found that methylation of this region of the *CASP8* gene was associated with *CASP8* silencing in both lung cancer (13) and pediatric tumor cell lines. To further investigate this relationship, we amplified two parts (319 bp and 456 bp) of this region that encompassed 15 CpG sites (and that included the MSP amplicon), and cloned the resultant amplicons. As demonstrated in Fig. 2*C*, cell lines BE(2)-M17 and D283, which lacked gene expression and had methylated bands by MSP, were heavily methylated at most CpG sites in all of the clones. Cell line SAOS-2, which only had an unmethylated band by MSP assay, completely lacked methylation at all sites. Cell line SK-N-FI, which had both methylated and unmethylated bands by MSP and was expression positive, demonstrated considerable clonal and site variation in its methylation pattern.

Expression of *CASP10* and *c-FLIP*. The *CASP10* gene is located ~20–30 kb 5′ of the *CASP8* gene (4) and *CASP10* expression has been reported in *CASP8*-negative pediatric tumors and cell lines (2, 7, 21). We have previously reported the absence of *CASP10* protein in lung and breast cell lines expressing mRNA. For these reasons, we compared mRNA and protein expression of these two functionally related and closely located genes (Fig. 2*A*). The intensities of expression of *CASP10* mRNA varied, although expression was present in all 13 cell lines examined. However, protein expression was present in only one cell line, SK-N-FI. *c-FLIP* resides in the same gene cluster at 2q33 as *CASP8* and *CASP10* (6). All of the cell lines expressed *c-FLIP* protein (Fig. 2*A*).

DISCUSSION

In this study, we demonstrated that methylation of *CASP8* was present in several types of pediatric tumors and its methylation was associated with gene silencing in pediatric tumor cell lines. Rhabdomyosarcomas (83%), medulloblastomas (81%), retinoblastomas (59%), and neuroblastomas (52%) were frequently methylated, whereas the frequencies of methylation were low or absent in other types of pediatric tumors. These results were not discrepant with previous reports (7, 9, 11, 12, 21–24) except for methylation in Ewing's sarcomas. Fulda *et al.* (12) showed that 13 of 20 Ewing's

sarcoma tumors were methylated, but in our study, methylation of *CASP8* was absent in Ewing's sarcoma tumors. However, Fulda *et al.* found protein expression (by immunostaining) in several of the methylated tumors, making their findings difficult to interpret. Methylation of *CASP8* was absent in corresponding nonmalignant tissues and in ganglioneuromas, which are considered to be a differentiated, benign form of neuroblastoma. These findings indicate that methylation of *CASP*8 is specific for certain pediatric tumors.

Recently, we found that the *RASSF1A* gene, a putative tumor suppressor gene, is frequently methylated in some types of pediatric tumors (18). We noted a high concordance rate between methylation of *CASP8* and *RASSF1A*. Concordant methylation of both genes may contribute to the pathogenesis of several types of pediatric tumors.

Because the MSP amplicon used in this and other studies does not target the promoter region of the gene, and because this site does not rigorously fulfill the criteria of a CpG island, we performed a detailed correlation between methylation, mRNA expression, protein expression, and protein function in pediatric cell lines. In general, there was a negative concordance between the presence of methylation (by MSP assay) and mRNA expression. Data from bisulfite-modified sequencing were consistent with these results. However, five cell lines had both methylated and unmethylated alleles, and four of these had mRNA expression, which indicated monoallelic inactivation with a functional unmethylated allele, or heterogeneity of methylation. Sequencing studies of one of these four cell lines demonstrated considerable clonal and positional variation in methylation. After treatment with 5-aza-CdR, expression of *CASP8* was restored in six of seven methylated cell lines lacking expression, but restoration was not detected after TSA treatment. These findings are further supportive evidence that methylation of *CASP8* was associated with gene silencing. Whereas methylation in the region examined is unlikely to be the direct cause of gene silencing, methylation in this region may reflect methylation in the promoter region.

Of 13 cells lines tested, there was concordance between expression of mRNA and protein, but protein expression was absent in 2 lines having mRNA expression. This finding suggests that posttranscriptional control may be responsible for gene silencing in some cases. On the other hand, there was good concordance between expression of protein and enzymatic activity of *CASP8*.

We also examined the state of the closely related *CASP10* gene. Although all of the 13 cell lines expressed mRNA, the intensity of the band was highly variable. However, by Western blot, only one cell line expressed protein. These findings are similar to our previous findings in lung and breast cancer cell lines (5). Teitz *et al.* (7) found variable intensities of protein expression in neuroblastoma cell lines. We have noted that some commercial antibodies cross-react with the similar-sized heat shock protein 60, including the antibody used by Teitz *et al.* (5), possibly accounting for this apparent discrepancy. *c-FLIP* is a molecule closely related to *CASP8* but proteolytically inactive and may function as an inhibitor of apoptosis. By Western blot, all of the cell lines expressed variable amounts of *c-FLIP*. There was no apparent relationship between expression of *CASP8*, *CASP10*, and *c-FLIP*.

We have demonstrated that the loss of protein expression and enzymatic activity of the *CASP8* gene are common in pediatric tumor cell lines. In many but not all cases, silencing appears to be related to methylation of the 5′ noncoding region of the gene. Methylation of the gene is common in several but not all types of pediatric tumors, and is concordant with methylation of the *RASSF1A* gene. Loss of expression of the closely related but functionally independent *CASP10* gene is also frequent in many pediatric tumor cell lines. Resistance to apoptosis is one of the hallmarks of cancer (25), and our findings suggest that deregulation of the death receptor pathway to apoptosis is frequent in many types of pediatric tumors and their cell lines.

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Medical Dictionary

4 entries found for **diagnosis**. Select an entry and then click 'Go'.

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differential diagnosis
DRG

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Main Entry: **di·ag·no·sis**
Pronunciation: ˌdī-ig-ˈnō-səs, -ˌg-
Function: *noun*
Inflected Form(s): *plural* **di·ag·no·ses** /-ˌsēz/
1 a : the art or act of identifying a disease from its signs and symptoms
b : the decision reached by diagnosis <a *diagnosis* of pneumonia>
2 : a concise technical description of a taxon

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Pronunciation Key

\&\ as **a** and **u** in abut	\ch\ as **ch** in chin	\o\ as **aw** in law
\ᵊ\ as **e** in kitten	\e\ as **e** in bet	\oi\ as **oy** in boy
\&r\ as **ur** and **er** in further	\E\ as **ea** in easy	\th\ as **th** in thin
\a\ as **a** in ash	\g\ as **g** in go	\th\ as **th** in the
\A\ as **a** in ace	\i\ as **i** in hit	\ü\ as **oo** in loot
\ä\ as **o** in mop	\I\ as **i** in ice	\u\ as **oo** in foot
\au\ as **ou** in out	\j\ as **j** in job	\y\ as **y** in yet
	\[ng]\ as **ng** in sing	\zh\ as **si** in vision
	\O\ as **o** in go	

For general laboratory use.
FOR *IN VITRO* USE ONLY.

Hexanucleotide Mix, 10 × conc.

For labeling DNA using random oligonucleotides as primers

Cat. No. 1 277 081
for 50 labeling reactions

Version 4, Feb. 2003
Store at −15 to −25° C

Product description

Contents

Sufficient for 50 standard labeling reactions.

Cat. No.	Label	Content
1 277 081	Hexanucleotide Mix 10x conc.	• 100 µl • 10x concentrated reaction buffer [0.5 M Tris-HCl, 0.1 M $MgCl_2$, 1 mM dithioerythritol (DTE), 2 mg/ml BSA, hexanucleotides, 62.5 A_{260} units/ml, pH 7.2 (20°C)]

Note: The mix is identical to that supplied in vial 5 of the DIG DNA Labeling and Detection Kit (Cat. No. 1 093 657) and of the DIG DNA Labeling Kit (Cat. No. 1 175 033), and in vial 6 of the Random Primed DNA Labeling Kit (Cat. No. 1 004 760).

Labeling principle

The method of „random primed" DNA labeling developed by Feinberg and Vogelstein (1,2) is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. All sequence combinations are represented in the hexanucleotide primer mixture, which leads to binding of primer to the template DNA in a statistic manner. Thus an equal degree of labeling along the entire length of the template DNA is guaranteed.

The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme, labeling grade. Modified deoxyribonucleoside triphosphates ([^{32}P]-, [^{35}S]-, [^{3}H]-, [^{125}I]-digoxigenin or biotin-labeled) present in the reaction are incorporated into the newly synthesized complementary DNA strand.

Application

Labeled DNA probes with high specific activity are used in a variety of hybridization techniques including:

- screening of gene libraries
- Southern and Northern blots
- *in situ* hybridizations

Sample material

- DNA fragments
- linearized plasmid DNA
- λDNA

Note: The length of the DNA fragments to be labeled does not influence the reaction. DNA fragments of 100 bp length are labeled equally well as linearized plasmid- or λ-DNA. The input DNA serves solely as template for the synthesis of labeled DNA, and is not degraded during the reaction, making it possible to label minimal amounts of DNA (10 ng) using this method.

Number of labeling reactions

The Hexanucleotide Mix can be used for 50 labeling reactions.

Quality control

The mix is tested for proper function in the Random Primed DNA Labeling Kit (Cat. No. 1 004 760) (by exchanging the mix for vial 6 of the kit).
Using the standard assay with 25 ng λDNA and 50 µCi [α-^{32}P]dCTP, 3000 Ci/mmol, an incorporation rate of > 55% = 1.6 × 10^9dpm/µg is obtained after 30 min incubation at 37°C.

Storage/ stability

The mix is stable at -15 to -25°C until the expiration date printed on the label.

Standard labeling

Additional required equipment and reagents

- Heating block or water bath
- Ice/water bath
- 0.2 M EDTA, pH 8.0
- Klenow enzyme labeling grade (Cat. No. 1 008 404)
- Desoxynucleotide-Triphosphate Set (Cat. No. 1 277 049); unlabeled dNTPs are also available as single nucleotides (please see Related products).
- Radioactive nucleotides. For radioactive labeling, we recommend [α-^{32}P]dCTP, 3000 Ci/mmol.

Preparation dNTP Stock Mix

To avoid pipetting mistakes, because of low volumes, prepare a stock mix of unlabeled dNTPs.

For labeling with radioactive dCTP mix „cold" dNTPs (dATP, dGTP and dTTP) 1:1:1.

Aliquots should be stored at -15 to 25°C. Repeated freezing and thawing should be avoided.

If another labeled dNTP than dCTP is used, „cold" dCTP has to be used instead of the corresponding labeled dNTP.

Procedure for labeling with [α-^{32}P] dCTP

Please refer to the following table.

Note: Larger amounts can be labeled by scaling up of all components and volumes.

Step	Action
1	The linearized DNA to be labeled should be purified by phenol/chloroform extraction and ethanol precipitation.
2	Add 25 ng DNA and autoclaved double dist. water to a final volume of 9 µl to a reaction vial.
3	Denature the DNA by heating in a boiling water bath for 10 min at 95°C and chilling quickly in an ice/water bath. ***Note***: Complete denaturation is essential for efficient labeling.
4	• Add the following to the freshly denatured probe on ice: **Reagent** / **Volume** **dNTP Stock Mix** — 3 µl **Hexanucleotide Mix** — 2 µl **50 µCi [α-^{32}P]dCTP,** 3000 Ci/mmol, aqueous solution — 5 µl **Klenow enzyme** — 1 µl • Mix and centrifuge briefly. • Incubate for 30 min to 20 h (overnight) at 37°C. ***Note***: Longer incubation can increase the yield of labeled DNA.
5	Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0) and/or by heating to 65°C for 10 min.

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Removal of unincorporated nucleotides

For the removal of non-incorporated deoxyribonucleoside triphosphates we recommened to use:

- Quick Spin Column, Sephadex G-50 (Fine) (Cat. No. 1 273 965) or
- repeated ethanol precipitation.

Degree of labeling

The degree of labeling is determined by comparison of incorporated to total input radioactivity in an aliquot of the reaction. The kinetics of the reaction may be followed by precipitation of the DNA with trichloroacetic acid of aliquots removed at various time points during the reaction.

Labeling assay with Digoxigenin-11-dUTP

Before you begin

For random priming of DNA with Digoxigenin, we offer more convenient products like for example DIG DNA Labeling Kit (Cat. No. 1175 033) or DIG-High Prime (Cat. No. 1585 606).

Additional required equipment and reagents

- Heating block or water bath
- Ice/water bath
- 0.2 M EDTA, pH 8.0
- DIG DNA Labeling Mix (Cat. No. 1 277 065)
- Klenow enzyme labeling grade (Cat. No. 1 008 404)

Procedure for labeling with DIG-11-dUTP

Very small amounts of probe cannot be used to detect rare sequences. Singl copy gene detection requires the entire probe from 300 - 1000 ng of template.

Note: Larger amounts can be labeled by scaling up of all components and volumes.

Step	Action
1	The linearized DNA to be labeled should be purified by phenol/chloroform extraction and ethanol precipitation.
2	Add 25 ng DNA and autoclaved double dist. water to a final volume of 15 µl to a reaction vial.
3	Denature the DNA by heating in a boiling water bath for 10 min at 95°C and chilling quickly in an ice/water bath. ***Note***: Complete denaturation is essential for efficient labeling.
4	• Add the following to the freshly denatured probe on ice:

Reagent	Volume
Hexanucleotide Mix, 10x conc.	2 µl
DIG DNA Labeling Mix, 10x conc.	2 µl
Klenow enzyme	1 µl

• Mix and centrifuge briefly.
• Incubate for 1 h to 20 h (overnight) at 37°C.
Note: Longer incubation can increase the yield of labeled DNA.

5	Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0)) and/or by heating to 65°C for 10 min.

Removal of unincorporated nucleotides

When the labeled DNA is used as hybridization probe, removal of unincorporated nucleotides is not necessary. However, if you like to remove non-incorporated Digoxigenin-11-dUTP use:

- Quick Spin Column, Sephadex G-50 (Fine) (Cat. No. 1 273 965)
- or repeated ethanol precipitation

References

1 Feinberg, AP. & Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, **132 (1)**, 6-13

2 Feinberg, AP. & Vogelstein, B. (1984) „A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity" Addendum. *Anal. Biochem.*, **137 (1)**, 266-7

Related products

Kits

Product	Pack size	Cat. No.
High Pure PCR Product Purification Kit	1 kit for 50 purifications	1 732 668
Agarose Gel DNA Extraction Kit	1 kit (max. 100 reactions)	1 696 505
DIG DNA Labeling and Detection Kit	1 kit	1 093 657
Random primed DNA Labeling Kit	1 kit (50 reactions)	1 004 760
High Prime DNA Labeling Kit	1 kit for 50 reactions	1 585 584
DIG DNA Labeling Kit	1 kit for 40 reactions	1 175 033
DIG Luminescent Detection Kit	1 kit for 50 blots	1 363 514

Songle reagents

Product	Pack size	Cat. No.
AMCA-6-dUTP	25 nmol	1 534 386
Biotin-16-dUTP	50 nmol	1 093 070
dATP, lithium salt	25 µmol (250 µl)	1 051 440
dCTP, lithium salt	25 µmol (250 µl)	1 051 458
Desoxynucleoside-Triphosphate Set	4x 10 µmol (100 µl)	1 277 049
dGTP, lithium salt	25 µmol (250 µl)	1 051 466
DIG DNA Labeling Mix	50 µl (25 reactions)	1 277 065
DIG-High Prime	160 µl (40 reactions)	1 585 606
Digoxigenin-11-dUTP alkali-stable	25 nmol	1 093 088
Digoxigenin-11-dUTP alkali-labile	25 nmol	1 573 152
dTTP, lithium salt	25 µmol (250 µl)	1 051 482
Fluorescein-12-dUTP	25 nmol	1 373 242
Quick Spin Columns for radiolabeled DNA purification, Sephadex G-50	20 columns	1 273 965
Tetramethyl-rhodamine-6-dUTP	25 nmol	1 534 378

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